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Genetic diversity among toxigenic and nontoxigenic *Vibrio cholerae* O1 isolated from the Western Hemisphere

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SUMMARY

Multilocus enzyme electrophoresis was used to examine genetic relationships among and between toxigenic and non-toxigenic isolates of *Vibrio cholerae* O1 obtained from patients and the environment in the US Gulf Coast and surrounding areas. A total of 23 toxigenic and 23 non-toxigenic strains were examined. All the toxigenic and 7 of the non-toxigenic strains had the same alleles at 16 enzyme loci, whereas the balance of the nontoxigenic strains had 9 distinct combinations of alleles. This study suggests that all of the toxigenic strains belong to a single clone, and that while some of the non-toxigenic isolates were related, most were of diverse origin.

INTRODUCTION

In the 62-year interval between 1911 and 1973, no cases of cholera were reported in the United States [1]. Unexpectedly, in 1973, an isolated case of cholera caused by *Vibrio cholerae* O1 occurred in Texas in a patient who had eaten seafood [1]. This case was followed by an outbreak in Louisiana in 1978 [2], two sporadic cases in Texas in 1981 [3, 4], and an outbreak on an oil-drilling platform on a bayou near the Texas coast in 1981 [5]. Since 1981 additional patient isolates have been obtained from Mexico in 1982 [6]; from Maryland in 1984 [7]; and from another outbreak in Louisiana in 1986 [8, 9]. Recently, an increasing number of reports have documented cholera in different geographic regions of the United States associated with the consumption of raw oysters obtained from the US Gulf Coast [10–12].

Many of these Gulf Coast isolates have been studied extensively and are biotype eltor, subserotype Inaba, produce cholera toxin (CT), are strongly haemolytic, and when tested, had a unique bacteriophage lysis pattern [6, 13, 14]. Furthermore,

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they have the same *Hin*d III restriction digest pattern that by Southern analyses has 2 fragments of approximately 6 and 7 kb which contain CT subunit A genes [6, 15] and an array of restriction fragments which hybridize in a unique pattern with vibriophage VcA-3 [16]. Eltor isolates from the Eastern Hemisphere and classical biotype isolates are easily distinguishable from the Gulf Coast isolates; CT-A genes are located on DNA fragments that are larger than 7 kb, and vibriophage genes, if present, are located on a different array of fragments within the *Hin*d III chromosomal digest [17, 18].

Since 1978, epidemiological and ecological studies of the Gulf Coast region have failed to identify a carrier or an environmental reservoir for the apparently endemic, toxigenic *V. cholerae* O1 [19]. During these investigations, several *V. cholerae* O1, including one isolate from Chesapeake Bay, Md. [20], have been identified that do not produce cholera toxin; studies using CT gene probes demonstrated that the isolates lacked chromosomal genes necessary for producing cholera toxin [18, 19]. In 1978, non-toxigenic *V. cholerae* O1 isolates were found to have phage sensitivity patterns that were different from patterns of the toxigenic isolates [14]. In 1980, a non-toxigenic strain of *V. cholerae* O1 was isolated from a patient with severe diarrhoea [21]. Since then, other non-toxigenic O1 strains have been reported to be isolated from cases of sporadic diarrhoeal disease [10, 22] usually associated with seafood from the Gulf Coast regions [23]. In addition, non-toxigenic strains have been isolated in Brazil, Guam, Mexico, and Peru [24–26]. Several studies have shown that both the non-toxigenic and toxigenic strains can grow in seafoods [4, 22, 23, 27].

The relationship between toxigenic and non-toxigenic strains is still not clear [16, 18, 19]. Probing with DNA from vibriophage VcA-3 revealed that toxigenic and some, but not all, non-toxigenic Gulf Coast isolates had the same VcA-3 integration site in the chromosome [16]. Additional studies are needed to show the relationship between toxigenic and non-toxigenic isolates of *V. cholerae* O1.

In recent years, multilocus enzyme electrophoresis (MEE) has been used to estimate the genetic diversity and structure in natural populations of *Escherichia coli* and a variety of other bacterial species [28, 29]. MEE has been used in several studies of *V. cholerae* O1 and non-O1 as well as other *Vibrio* species. These studies examined only a few enzymes and few isolates [30–33], did not specifically address the relationships within the *V. cholerae* O1 group, and did not include isolates from the Gulf of Mexico region.

The purpose of this study was to use MEE to examine genetic relationships among and between toxigenic and non-toxigenic isolates of *V. cholerae* O1 obtained from patients and the environment in the Western Hemisphere.

METHODS

Bacterial isolates

A total of 46 *V. cholerae* O1 isolates from patients or the environment within the Western Hemisphere were selected. All isolates are *V. cholerae* O1, and include 23 toxigenic and 23 non-toxigenic strains. All isolates were previously tested for toxin production by enzyme-linked immunosorbent assay (ELISA), a latex aggluti-

nation kit (VET-RPLA, Oxoid USA, Inc., Columbia, Md.*), and CT DNA probes [34]. A detailed description of each isolate is presented in Table 1.

Enzyme extract preparation

Strains were grown overnight at 37 °C in 100 ml brain heart infusion broth (Becton Dickinson & Co., Cockeysville, Md.) in a shaking water bath. The cells were harvested by centrifugation and suspended in 2·0 ml of buffer solution (10 mM-Tris-HCl, pH 6·8, containing 1 mM-EDTA and 0·5 mM-NADP) and 1·5 ml of glass beads (75–150 μm, G-2381, Sigma Chemical Co., St Louis, Mo.). The suspensions were held in an ice bath for 45 min, and then the cells were lysed by mixing at a high speed on a heavy-duty vortex mixer (American Scientific Products, McGaw Park, Ill.) at 1-min intervals for a total of 3 min. Cell debris and glass beads were removed by centrifugation at 20000 g for 20 min followed by filtration of the supernatant with a 0·22 μm, Millex-GV filter (Millipore Corp., Bedford, Mass.). Aliquots of the filtrates were stored at −70 °C.

Electrophoresis and enzyme analysis

Techniques of starch gel electrophoresis and enzyme staining were similar to those described by Selander and co-workers [29], as modified by Woods and co-workers [35]. NADPH and NADH diaphorase were stained by the methods of Harris and Hopkinson [36]. A total of 15 enzymes were assayed (Table 3).

A total of six buffers and buffer combinations listed in Selander and colleagues [29] were tested for the best resolution of the enzyme extracts from a minimum of 25 diverse isolates of *V. cholerae*. Two buffers provided the best resolution. Potassium phosphate buffer (electrode buffer pH 6·7, gel buffer pH 7·0) was used for electrophoresis of MDH, ME, G6P, GOT, GPT, LAP, and PGI, and Tris-citrate buffer (electrode and gel buffer both were pH 8·0) for THD, ALD, LDH, ADK, IDH, NSP, DA-NADPH, and DA-NADH. A key to enzyme nomenclature is given in Table 3.

Electrophoretic variants (electromorphs which correspond to specific alleles), of each enzyme were distinguished by differences in the migration rate of specifically stained protein bands that were numbered in order of increasing rate of anodal migration. Isolates that lacked an enzyme were assigned a null allelic state at the locus in question.

NADPH diaphorase was present as two electromorphs or two bands in most strains. Each band was considered to be a separate enzyme locus for a total of 16 enzyme loci (Tables 2 and 3).

Statistical analysis

For analytic purposes, electromorphs of an enzyme were equated with alleles at the corresponding structural gene locus so that each bacterial strain was fully characterized by its multilocus genotype (allele combination) for the enzyme-encoding loci assayed. Genetic diversity at each enzyme locus (the degree to which each enzyme locus varied) was calculated as described by Selander and colleagues [29, 37]. Distinctive multilocus genotypes were designated electrophoretic types

* Use of trade names and commercial sources is for identification only and does not imply endorsement by the Public Health Service or by the US Department of Health and Human Services.

Table 1. *Characteristics of 46 strains of* Vibrio cholerae *O1*

ET*	Strain number	Year of isolation	Biotype	Sub-serotype	Source	Location	Toxigenic‡
1	3242–73	1973	Eltor	Inaba	Stool	Tex., USA	+
1	2164–78	1978	Eltor	Inaba	Stool	La., USA	+
1	2468–78	1978	Eltor	Inaba	Shrimp	La., USA	+
1	2559–78	1978	Eltor	Inaba	Crab	La., USA	+
1	2631–78	1978	Eltor	Inaba	Bayou	La., USA	+
1	2741–80	1980	Eltor	Inaba	Stool	Fla., USA	–
1	1064–81	1981	Eltor	Inaba	Stool	Tex., USA	+
1	1428–81	1981	Eltor	Inaba	Stool	Tex., USA	+
1	2373–81	1981	Eltor	Inaba	Stool	Tex., USA	+
1	776–83	1983	Eltor	Inaba	Stool	N.J., USA/ Mexico	+
1	917–84	1984	Eltor	Inaba	Stool	Ga., USA	–
1	1267–84	1984	Eltor	Inaba	Stool	Md., USA	+
1	2496–85	1985	Atypical	Inaba	Stool	Fla., USA	–
1	2469–86	1986	Eltor	Inaba	Stool	La., USA	+
1	2489–86	1986	Eltor	Inaba	Stool†	Fla., USA	+
1	2496–86	1986	Eltor	Inaba	Sewage	La., USA	–
1	2497–86	1986	Eltor	Inaba	Sewage	La., USA	–
1	2512–86	1986	Eltor	Inaba	Sewage	La., USA	+
1	2514–86	1986	Eltor	Inaba	Sewage	La., USA	+
1	2523–86	1986	Eltor	Inaba	Sewage	La., USA	+
1	2538–86	1986	Eltor	Inaba	Stool†	Ga., USA	+
1	2583–87	1987	Eltor	Ogawa	Stool	Peru	–
1	2584–87	1987	Eltor	Ogawa	Stool	Peru	–
1	2514–88	1988	Eltor	Inaba	Stool†	La., USA	+
1	2520–88	1988	Eltor	Inaba	Stool†	Colo., USA	+
1	2529–88	1988	Eltor	Inaba	Stool†	Mo., USA	+
1	2537–88	1988	Eltor	Inaba	Stool†	Md., USA	+
1	2542–88	1988	Eltor	Inaba	Stool†	Tex., USA	+
1	2557–88	1988	Eltor	Inaba	Stool†	Nev., USA	+
1	2561–88	1988	Eltor	Inaba	Stool†	Ga., USA	+
2	2479–86	1986	Eltor	Inaba	Sewage	La., USA	–
3	3272–78	1977	Eltor	Inaba	Bay	Md., USA	–
4	2171–81	1981	Atypical	Inaba	Water	Fla., USA	–
5	1062–80	1980	Eltor	Ogawa	Bayou	La., USA	–
6	2168–81	1981	Atypical	Ogawa	Water	Fla., USA	–
7	1029–84	1984	Eltor	Inaba	Stool	Fla., USA	–
7	2452–88	1988	Eltor	Inaba	Blood	Miss., USA	–
7	2469–88	1988	Eltor	Inaba	Hip wound	Miss., USA	–
8	2483–85	1985	Eltor	Inaba	Stool	Cal., USA	–
8	1165–77	1977	Atypical	Inaba	Gallbladder	Ala., USA	–
8	692–79	1979	Atypical	Inaba	Canal	La., USA	–
8	1077–79	1979	Atypical	Inaba	Leg wound	La., USA	–
8	884–82	1982	Atypical	Inaba	Stool	Fla., USA	–
9	468–83	1983	Eltor	Ogawa	Stool	Pa., USA/ Mexico	–
10	1074–78	1978	Atypical	Ogawa	Sewage	Brazil	–
10	2633–78	1978	Atypical	Ogawa	Sewage	Brazil	–

* Electrophoretic type.
† Ate raw oysters from Gulf Coast region.
‡ Reactions are based on ELISA and CT DNA probe assays [34].

Table 2. *Allele profiles in 10 electrophoretic types (ETs)*

		ENZYMES															
ET	No. of isolates	THD	IDH	ADK	MDH	ME	G6P	GPT	GOT	LAP	PGI	ALD	LDH	NSP	DA1	DA2	DA3
1	30	3	2	3	1	3	4	3	2	2	1	1	1	2	2	1	1
2	1	3	2	3	1	3	3	3	2	2	2	1	1	2	2	1	1
3	1	2	2	2	1	3	3	3	2	2	2	1	1	2	2	1	1
4	1	3	2	2	1	3	2	3	2	2	2	1	1	2	2	1	1
5	1	6	2	3	1	2	4	3	2	2	3	1	1	2	2	1	1
6	1	5	2	3	1	2	3	3	1	2	3	1	1	2	2	1	1
7	3	3	2	3	2	3	4	3	3	2	1	1	2	2	2	1	1
8	5	3	2	3	2	3	3	3	3	2	1	1	2	2	2	1	1
9	1	3	1	2	1	1	3	3	2	2	3	1	1	2	2	1	2
10	2	3	3	2	1	3	3	3	1	2	3	1	1	2	2	1	2

See Table 3 for key to enzymes.

(ETs) and were numbered by their inferred relationships from a cluster analysis. Mean genetic diversity per locus was calculated as the arithmetic average of the values for all of the loci. Genetic distances between pairs of strains were calculated as the proportion of weighted mismatches of alleles, and a dendrogram showing the clustering or relatedness of the ETs was generated by the unweighted pair group method for arithmetic averages (UPGMA) [38], using the commercial program CLUSTAN 1C [39].

RESULTS

Among 16 enzyme loci, 10 were polymorphic. All 46 isolates were typable by this procedure. Ten distinct combinations of alleles (ETs) were found (Table 2). To analyse the genetic relatedness among all 46 strains, we calculated a matrix of pairwise unweighted similarity coefficients and produced a dendrogram (Fig. 1) by the average linkage method of clustering. The smallest genetic distance in the dendrogram (0·04) corresponds to a single-locus difference between ETs, and the largest distance (0·292) corresponds to an average difference at 6 of the 16 loci assayed. The number of alleles for isolates ranged from 1 for GPT, LAP, ALD, NSP, DA1, and DA2 to 4 for THD, with an average diversity of 2·1 (Table 3). The average genetic diversity among ETs was 0·31.

The dendrogram in Fig. 1 consists of 4 clusters of 2 or more closely related ETs diverging at genetic distance of less than 0·12. Clonal genotypes grouped at this level of genetic distance exhibit allelic difference, on average, at 5 or fewer of the 16 enzyme-encoding loci.

Cluster A is composed of 4 clones (ET-1, 2, 3, 4). The 23 Gulf Coast toxigenic strains fall into ET-1. ET-1 also contains 7 nontoxigenic strains from stool, sewage and the environment, including 2 stool isolates from Peru. ET-2, 3, and 4 contain 1 isolate per clone and all are subserotype Inaba. ET-2 is an atypical sewer isolate, while ET-3 and ET-4 are environmental isolates.

Cluster B is composed of ET-5 and ET-6, each contain an environmental isolate of the Ogawa subserotype; ET-5 is the eltor biotype and ET-6 is atypical.

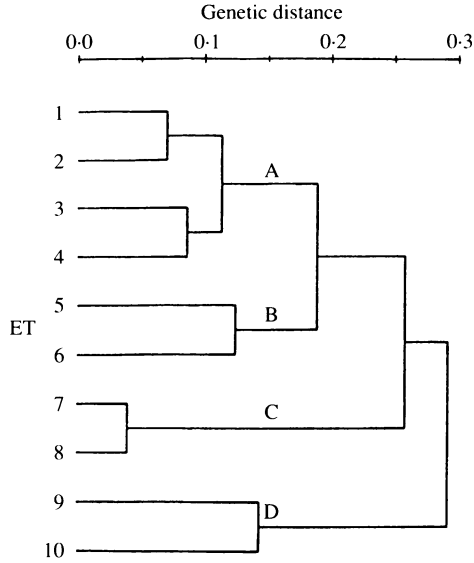

Fig. 1. Genetic relationships among 10 ETs of 46 strains of *Vibrio cholerae* O1. The dendrogram was produced by the average-linkage methods of clustering from a matrix of coefficients of weighted distance, based on 16 enzyme loci. The ET numbers are those shown in Table 1.

Table 3. *Genetic diversity of 16 enzyme loci, assayed by enzyme electrophoresis of 46 strains of* Vibrio cholerae *O1*

E.C. No.	Symbol	Enzyme	No. of alleles	Genetic diversity
1.1.1.x	THD	Threonine dehydrogenase	4	0·53333
1.1.4.42	IDH	Isocitrate dehydrogenase	3	0·37778
2.7.4.3	ADK	Adenylate kinase	2	0·53333
1.1.1.37	MDH	Malate dehydrogenase	2	0·35556
1.1.1.40	ME	Malic enzyme	3	0·51111
1.1.1.49	G6P	Glucose 6-phosphate dehydrogenase	3	0·60000
2.6.1.2	GPT	Glutamic-pyruvic transaminase	1	0·00000
2.6.1.1	GOT	Glutamic-oxalacetic transminase	3	0·62222
3.4.1.1	LAP	Leucine aminopeptidase	1	0·00000
5.3.1.9	PGI	Phosphoglucose isomerase	3	0·73333
1.4.1.1	ALD	Alanine dehydrogenase	1	0·00000
1.1.1.27	LDH	L-Lactate dehydrogenase	2	0·35556
2.4.2.1	NSP	Nucleoside phosphorylase	1	0·00000
1.6.2.2	DA1	NADPH Diaphorase	1	0·00000
	DA2	NADPH Diaphorase	1	0·00000
1.6.2.2	DA3	NADH Diaphorase	2	0·35556
Average			2·1	0·31111

Cluster C contains 2 ETs and is a mixture of eltor and atypical strains. ET-7 has 3 eltor strains; ET-8 contains 1 eltor and 4 atypical strains. The 4 extraintestinal patient isolates of *V. cholerae* O1 are all in this cluster, 2 in ET-7, and 2 in ET-8. Seven of the 8 isolates in this cluster were from patients.

Cluster D contains 2 ETs. ET-9 contains 1 isolate from a U.S. patient who travelled to Mexico. ET-10 contains 2 atypical strains isolated from a sewer in Brazil. The difference between ET-9 and ET-10 is 3 loci, the genetic distance is about 0·143. All 3 strains are related to other *V. cholerae* O1 in this study by a

genetic distance of about 0·292, indicating a greater genetic difference from other study isolates. The *V. cholerae* O1 strains in this cluster are all serotype Ogawa.

DISCUSSION

All toxigenic *V. cholerae* O1 eltor isolates from the Western Hemisphere appear to be related and are found in ET-1. The repeated recovery of isolates of the same multilocus genotype at many different locations around the Gulf Coast and over a period of 15 years strongly suggests an endemic problem due to one *V. cholerae* O1 clone. It supports the hypothesis that the strain entered the Gulf Coast region before 1973 when the first case of cholera since 1911 was reported in Texas. The occurrence of nontoxigenic strains, otherwise indistinguishable from the toxigenic clone, leads to the speculation that these strains gain or lose toxin genes. The inverted repeat DNA sequences flanking toxin genes also support this hypothesis [40]. One organism in this group, 2489–86, has been reported to contain only one of the two toxin gene copies normally present in the toxigenic clone [41].

Our findings indicate that the nontoxigenic *V. cholerae* O1 strains are a heterogeneous group spanning the genetic distance from ET-1 through ET-10. Similarly, the nontoxigenic strains are isolated from both stool and extraintestinal sites, indicating a rather diverse disease potential by association. It is interesting that the one toxigenic *V. cholerae* O1 strain in this study from Mexico, 776–83, is in ET-1 with the toxigenic *V. cholerae* O1 US isolates. The nontoxigenic strains from Peru are also in ET-1, but the non-toxigenic strains from Brazil are in the least related group, ET-10. The findings also indicate that no genetic difference exists between isolates from human and from environmental sources. Distribution of electromorphs in human and environmental isolates was quite similar for most of the loci. The high degree of genotypic and phenotypic similarity between human and environmental isolates is consistent with *V. cholerae* O1 survival and persistence in the environment. A recent report from Australia also indicated that toxigenic *V. cholerae* O1 from humans and from the environment belong in the same zymovar [30].

Before 1986, most of the cholera cases caused by toxigenic Gulf Coast isolates were associated with consumption of undercooked crab and shrimp dishes [1, 2, 23]. The first cholera case clearly correlated with raw oyster consumption occurred in 1986 [41], followed by additional cases associated with eating raw oysters in 1986 and 1988 [12]. We found that all of these isolates as well as other Gulf Coast toxigenic isolates before or after 1986 are in ET-1. It seems apparent that *V. cholerae* O1, toxigenic eltor strains can survive and/or multiply in shellfish such as crab, oyster, and shrimp [23]. Raw oysters have been the vehicles of transmission not only for pathogens of faecal origin, such as hepatitis A virus and *Salmonella typhi*, but also for several pathogenic free-living *Vibrio* species, including nontoxigenic *V. cholerae* O1, non-O1 *V. cholerae*, *V. vulnificus*, *V. mimicus*, *V. hollisae*, and *V. parahaemolyticus* [11, 27].

Because oysters are frequently eaten raw, they represent a potentially important and newly recognized vehicle of infection. Although the risk is probably small, oysters must now be considered a potential vehicle for cholera [11, 12, 41].

The four extraintestinal isolates are all nontoxigenic O1 strains. In this study, they are in one cluster, but in different ETs. Two Mississippi eltor isolates in 1988

are in ET-7; two other Alabama and Louisiana isolates in 1977 and 1979 are in ET-8. At least one other report has appeared of nontoxigenic *V. cholerae* O1 strains apparently causing extraintestinal human illness [10]. The four extraintestinal isolates described in this study may possess some unknown mechanism of disease production.

In previous studies, atypical *V. cholerae* O1 isolates have often been included in the eltor biotype [10, 25, 26, 42], implying that atypical strains are similar to the eltor biotype strains. Our results show that atypical and eltor biotype strains were present in both ET-1 and ET-8; otherwise genetic diversity apparently exists within both groups. The subserotypes also occur in several diverse ET groups. These findings indicate the lack of clonal discrimination offered by the routine laboratory tests for phenotypic characteristics. The findings likewise indicate the value of enzyme electrophoresis in determining some of the genetic and epidemiologic relationships among *V. cholerae* O1 strains. Many of the relationships correlate with molecular genetic analysis such as restriction endonuclease patterns as well as phage and toxin gene hybridization patterns [15, 16, 18].

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